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To test the hypothesis that the signaling protein Rac3 is critical for the initiation of breast cancer metastasis, we created stable fluorescently-tagged breast cancer cell lines that express active and inactive forms of Rac3 and its close homolog Rac1 in a highly metastatic variant of the MDA-MB-435 metastatic breast cancer cell line. Dominant active forms of Rac3 and Rac1 were stably expressed in a low metastatic variant of MDA-MB-435. High metastatic cell lines expressing dominant negative Rac1 or Rac3 demonstrated similar reduction in invasion and migration *in vitro* compared to controls. Cell lines expressing a dominant negative Rac1 or Rac3 did not show differences in proliferation; however, when implanted in nude mice, both cell lines produced smaller primary tumors that demonstrated reduced metastasis compared to controls. Expression of dominant active Rac1 or Rac3 in a low metastatic breast cancer cell variant resulted in enhanced migratory and invasive properties. Interestingly, the low metastatic breast cancer cell lines expressing a dominant active Rac3 were more invasive *in vitro* than the same cell line expressing similar levels of dominant active Rac1. These cells are currently being analyzed in the nude mouse model of experimental metastasis. To provide a direct assessment of these mutant cell lines, we have developed a fluorescence illumination system and are adapting a fluorescence confocal microscope to specifically image fluorescence breast cancer cells in live mice.

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Table of Contents

Introduction

Cancer metastasis is a complex multistep process in which malignant cells escape from a primary tumor, invade surrounding tissue, migrate through the extracellular matrix, and are transported via the circulatory system to establish secondary tumors at distant sites (1-3). Rho family of small GTPases are key regulatory molecules that have been implicated in cell invasion (4; 5). The small GTPase Rac3 is a closely related homologue of the Rho GTPases Rac1 and Cdc42, which have been shown to regulate actin cytoskeletal reorganization during cell invasion (6). Rac3 is constitutively active in aggressively dividing, high metatsatic breast cancer cell lines and tissues. Transient expression of dominant active Rac3, activated DNA synthesis and conferred a highly proliferative phenotype to human mammary epithelial cells via activation of a downsteam effecctor, P21-activated kinase (PAK) (7). PAKs are a group of 62-68 kDa serine/threonine kinases that have been identified as targets of activated Rac and Cdc42 (8-11). PAKs have been implicated in the regulation of the stress-activated MAP kinases, p38 and JNK, cytoskeletal rearrangement, cell-extracellular matrix interactions, cell motility, and apoptosis (9; 12-14). In vitro studies using breast cancer cell lines have shown that PAK regulates anchorage-independent growth, mitotic spindle organization, tumorigenicity, and angiogenesis as well as cytoskeletal reorganization, cell migration, and invasion (15-19). These are all cell functions that are expected to be dysregulated in metastatic cancer. However, a role for PAK and Rac3 as metastasis promoters *in vivo* has yet to be substantiated.

Hypothesis

We hypothesize that the signaling proteins Rac3 and PAK are critical for the initiation of metastasis.

Results

Vector construction

Rac1 or Rac3 cDNA containing aminoacid substitutions that render the expressed protein dominant negative (T17N), or dominant active (G12V) were cloned into bicistronic vectors that express red fluorescent protein (RFP) and neomycin resistance (Clontech). The cloning of these vectors was described in the 2003 annual report.

Cell Lines

Stable breast cancer cell lines expressing bicistronic vectors containing active and inactive forms of Rac1 or Rac3 and RFP have been created. The annual report for 2003 described the creation of these cell lines and demonstration of the predicted Rac activities. We reported the expression of a dominant active Rac3 in the Hs578t primary breast cancer cell line, which was selected as a model system for a non-metastatic cell line. Since then, we have experienced unforeseen difficulties with establishment of primary tumors in the mammary fat pads of nude mice using the Hs578t parental cell line.

Therefore, to ensure primary tumor establishment with parental cells and to obtain a better assessment of the effect of expressing Rac3 mutants in a similar genetic background, we created Rac3 or Rac1 mutants in metastatic variants of the same MDA-MB-435 cell line. These variants were created according to their relative metastatic efficiency in nude mice by our collaborator Dr. Janet Price (UT-MDACC, Houston, TX). We have analyzed the Rac expression and activity of these cell lines using a pan Rac antibody. The panel of MDA-MB-435 metastatic variants (four cell lines) expressed similar levels of Rac proteins. However, Rac activity, as

monitored according to interaction of the endogenous Rac proteins with a GST-PBD (p21-binding) domain of PAK that specifically binds to the GTP bound active form of Rac, correlated with metastatic efficiency (Fig. 1). Methods as described in (7; 20). Therefore, we selected the high metastatic MDA-MB-435 α6HG6 variant with high Rac activity and the low metastatic MDA-MB-435Br variant with little to none Rac activity for further analysis.

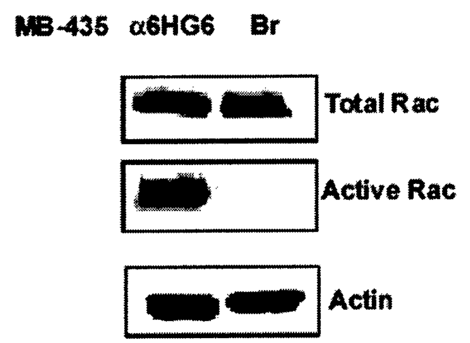


Figure 1. Rac activity in high metastatic MDA-MB-435 α6HG6 variant and low metastatic MDA-MB-435Br variant. Whole cell lysates of MDA-MB-435α6HG6 or MDA-MB-435Br were incubated with PAK-PBD Protein GST Beads (Cytoskeleton Inc., CO) and subsequently pelleted. Pellets were dissolved in sample buffer, run on SDS-PAGE, and western blotted for Rac using a pan Rac antibody (Active Rac). Equal loading was maintained by performing a total protein assay and demonstrated by western blot analysis of whole cell lysates for total Rac or actin.

These cell lines were stably transfected with Rac1 or Rac3 mutant constructs and the following cell lines were created:

1. MDA-MB-435 α6HG6 expressing dominant negative Rac3(T17N) and RFP.
2. MDA-MB-435 α6HG6 expressing dominant negative Rac1(T17N) and RFP.
3. MDA-MB-435 α6HG6 expressing RFP only.
4. MB-435 Br expressing dominant active Rac3(G12V) and RFP.
5. MB-435 Br expressing dominant active Rac1(G12V) and RFP.
6. MB-435 Br expressing RFP only.

Characterization of mutant breast cancer cell lines

1. ***In vitro characterization of MDA-MB-435.α6HG6 variant expressing dominant negative (T17N) Rac3 or Rac1***

Cells expressing Rac3 or Rac1 (T17N) were analyzed for alterations in their motile and invasive potential in response to extracellular matrix components. Modified Boyden chambers (tissue culture treated, 6.5 mm diameter, 10μm thickness, 8 μm pores, Transwell®, Costar Corp., Cambridge, MA) were coated on the underside (haptotactic migration), or the upper surface (invasion), of the membrane with matrigel (Fisher Scientific, TX), 50 μg/ml laminin, or 50 μg/ml fibronectin (Gibco BRL, MD) overnight at 4° and then placed into the lower chamber containing 500 μl culture media with 10% fetal bovine serum (FBS). Serum starved cells (10^5 cells) were added to the upper surface of each migration chamber and allowed to migrate to the underside of the membrane for 4 hours (migration) or 24 hours (invasion). The non-migratory cells on the upper membrane surface were removed with a cotton swab, the migratory cells attached to the bottom surface of the membrane stained with propidium iodide (CalBioChem-Novabiochem Corp., CA) and quantified. Expression of a dominant negative Rac1 or Rac3 in this high metastatic breast cancer cell variant resulted in reduced cell migration or invasion (Fig. 2). Invasive and migratory responses were similar to expression of equal amounts of dominant negative Rac1 or Rac3.

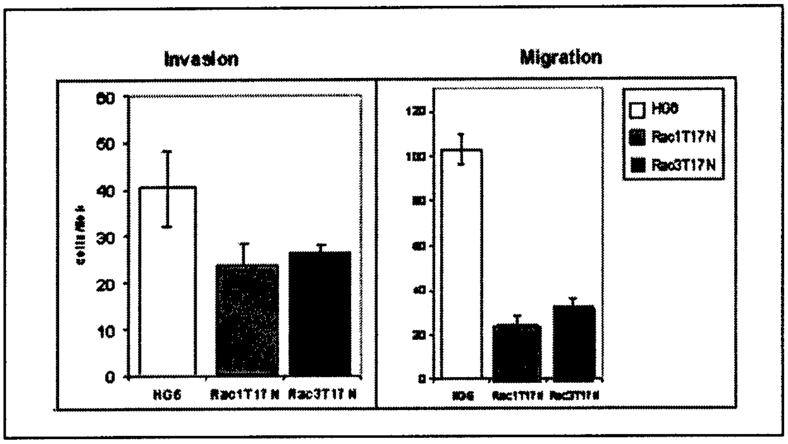


Figure 2. Invasion and migration assays of control (HG6), Racl(T17N), or Rac3(T17N) expressing MB-435 cells. Quiescent MB435.α6HG6 (high metatstatic), MB435.α6HG6.RFP (vector control), MB435.α6HG6 Racl(T17N), or MB435.α6HG6 Rac3(T17N) breast cancer cell lines were plated onto the membrane of a Transwell (Costar, MA). Invasion assays across laminin or migration towards laminin was quantified. Y-axis represents the number of cells/microscopic field for at least 10 microscopic fileds/cell line. Error bars represent +/- SEM.

2. ***In vivo characterization ofMDA- MB-435.α6HG6 variant expressing dominant negative (T17N) Rac3 or Racl***

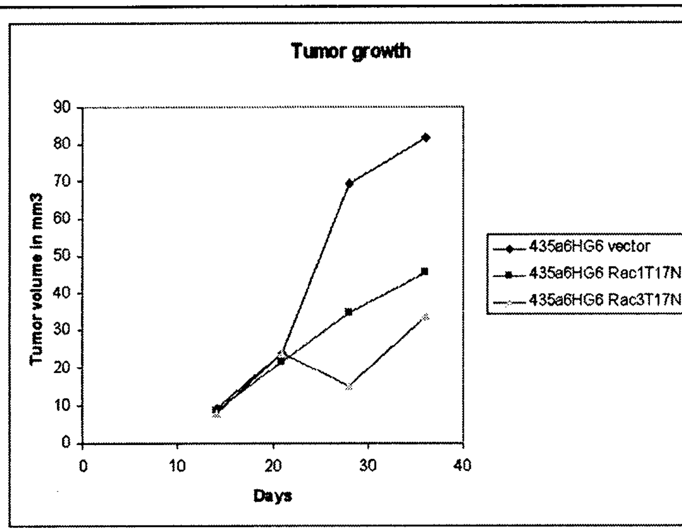


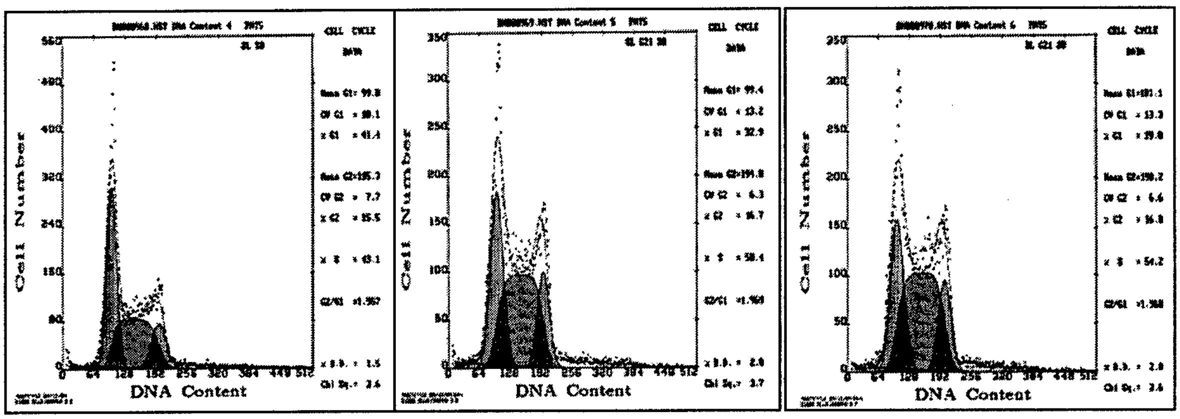
Figure 3. Primary tumor growth of MB-435 high metastatic breast cancer cells expressing Racl (T17N) or Rac3(T17N). Approximately 0.1 ml of about 1X10^6 MB-435a6HG6 cells expressing vector alone, Racl(T17N) or Rac3 (T17N) cells were injected into the mammary fat pad of anesthetized female nude mouse and examined daily for tumor formation. Results are representative of 6 mice per treatment.

We have initiated an *in vivo* analysis of MB435.α6HG6 cells expressing Racl or Rac3(T17N) to investigate whether dominant negative Rac3 expression affects metastatic efficiency in the nude mouse model. We successfully created primary breast tumors using the control MB-435.α6HG6 variant. Contrrol, Racl(T17N), or Rac3(T17N) expressing cells were injected into the mammary fat pad of female nude mice. Twenty days following inoculation, both Racl(T17N) and Rac3(T17N) expressing tumors were similar in size. However, these

tumor sizes were significantly smaller than the primary breast tumors created by the control cell line (Fig. 3). We are currently analyzing these mice for potential differences in metatstaic potential.

3. *In vitro characterization of MDA-MB-435.α6HG6 variant expressing dominant negative (T17N) Rac3 or Rac1*

Next, we created stable cell lines of the low metastatic MDA-MB-435Br variant expressing dominant active Rac1 or Rac3. The altered activity of mutant Rac3 proteins in the stably transfected cell lines was also confirmed by analysis of Rac3 activity using the *in vivo* assay described in (7; 20). The control cells did not demonstrate any Rac3 activity (data not shown). Expression of a dominant active Rac1 or Rac3 increased Rac1 and Rac3 activity but did not change the proliferative efficiency of MDA-MB-435Br cells.



MDA-MB-435.Br **Rac1(G12V)** **Rac3(G12V)**

Figure 4. FACS (fluorescence activated cell sorter) analysis of MDA-MB-435Br cells expressing vector alone, Rac1(G12V), or Rac3(G12V). Cells growing in serum were stained with propidium iodide (PI) to visualize nuclei and subjected to FACS analysis. The DNA content was analyzed by fluorescence of PI.

As expected, cells expressing dominant active forms of Rac1 or Rac3 demonstrate a more motile phenotype. When cells were stained with rhodamine phalloidin for F-actin and with an antiphosphotyrosine antibody followed by a FITC-tagged secondary antibody to visualize focal adhesions, the dominant active Rac1 or Rac3 expressing cells demonstrated more membrane ruffles and focal adhesions (Fig. 5).

4. In vivo image analysis

The overall focus of this proposal is to develop and establish direct *in vivo* imaging for the functional analysis of signaling proteins involved in breast cancer metastasis. To this end we are adapting a confocal fluorescence microscope to image live animals with fluorescent breast tumors. This microscope is still under construction. We have installed special fluorescent filters enable image analysis at wave lengths ranging from 400-600nnm. Currently, we are adjusting the dichoric mirrors to obtain better resolution.

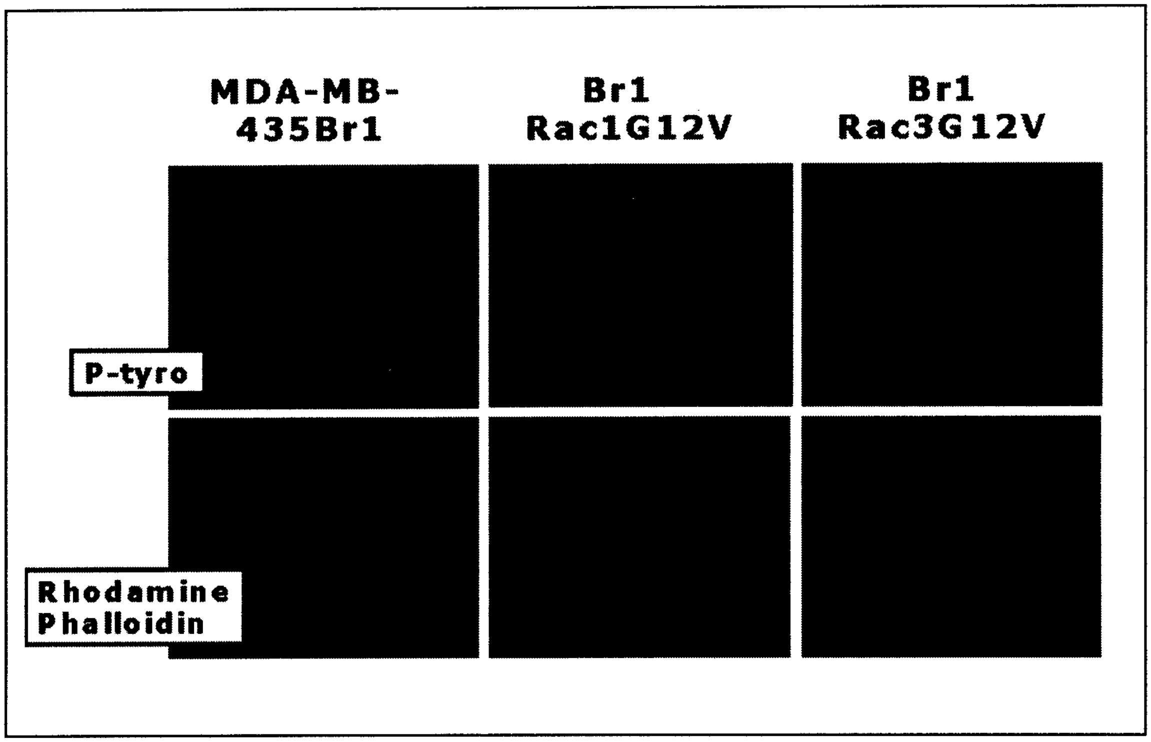


Figure 5. Phenotype of MB435Br low metastatic cells expressing Rac1 and Rac3 mutants. Top row, cells stained for focal adhesions using an anti phosphor-tyrosine antibody followed by FITC-secondary antibody. Bottom row, cells stained for F-actin with rhodamine phalloidin.

Prior to image analysis in the confocal microscope, we have initiated analysis of fluorescent tumors using an illumination system that was developed specifically to image grow tumor take and progression in nude mice. This system consists of a light source, filtered to provide optimal tissue illumination; an imaging device; and a filter used to create optimal observation conditions.

We have initiated a preliminary investigation to assess the exact kinetics at which the invasive breast cancer cells leave the primary tumors created by high metastatic MDA-MB-435 variant (α6HG6) expressing RFP (Fig. 6). Image analysis was started directly at the time of injection of $1X10^6$ RFP-tagged and followed tumor take, establishment of primary tumors, and metastatic progression (Fig. 7).

The fluorescence emission from live mice was recorded using a Canon EOS-D30 digital camera, where an emission filter is attached to the camera lens to reject scattered or reflected excitation light. The camera has a sensor size of 22.7 x 15.1 mm, and has options of acquiring images that are 1440 x 960 pixels or 2160 x 1440 pixels. When the entire imaging system is taken into account (including the optics of the camera), with 350 mm distance between the front lens element and the specimen, the pixel size becomes 25.6 microns / pixel. At these settings, in order to resolve two objects, according to theoretical calculations (Nyquist theorem), the objects must be approximately 59 microns apart, which is adequate for our needs.

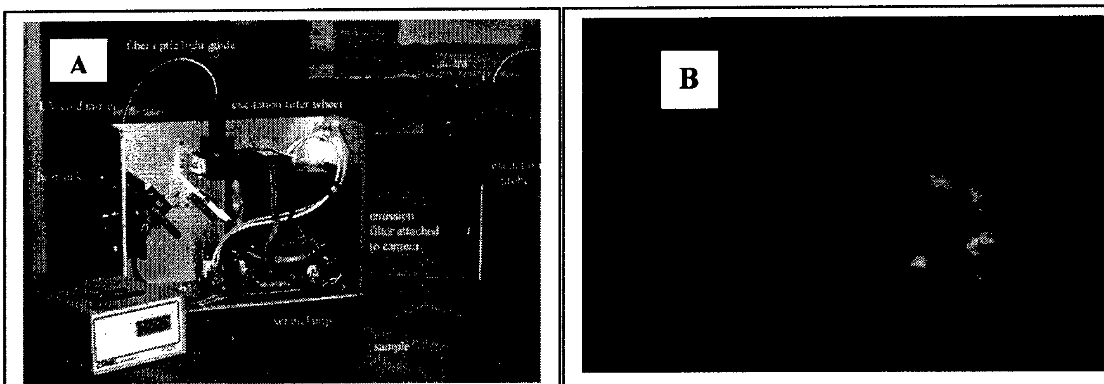


Figure 2. Picture of vision enhancement system setup [1].

Figure 6. Illumination system for macroscopic fluorescence image analysis. A. An illumination system that conatins a fiber optic light source connected to excitation and emission filters for green and red fluorescence detection has been adapted for image analysis of fluorescent tumors. **B.** A shaved scid mouse with primary and secondary tumors as viewed under the illumination system. The mouse was placed directly underneath the camera with attached emission filters and the fluorescence at 580nm was digitally recorded and analyzed.

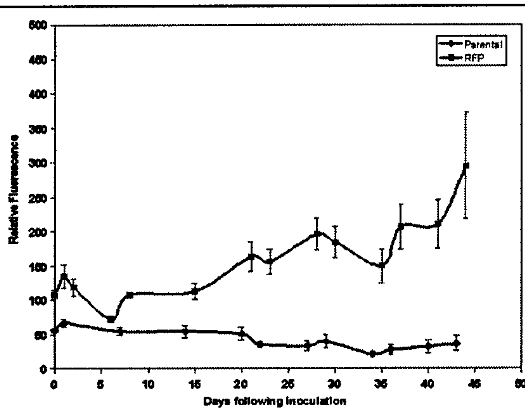


Figure 7. Relative fluorescence of RFP-tagged breast cancer cells following inoculation. $1X10^6$ RFP-tagged and parental control MDA-MB-435 cells were inoculated into left and right mammary fat pads (respectively) of a female nude mouse. Starting from time of inoculation, tumor take and progression of the primary tumors were monitored using the fluorescence illumination system. Fluorescence intensity of primary tumors from RFP-tagged (red line) or parental (blue) was determined every other day for 45 days.

Key Research Accomplishments

	In vitro **mutant analysis**	*In vivo* **mutant analysis**
1. MDA-MB-435.α6HG6 (high metastatic breast cancer variant) expressing dominant negative form (T17N) of Rac1 or Rac3.	Western analysis, Rac1 and Rac3 activity assays, invasion, and migration assays completed.	Primary tumorigenesis analyzed. *In vivo* image analysis of metastasis under study.
2. A low metastatic variant of the MB-435 cell line (MB-435 Br) expressing dominant active (G12V) form Rac1 or Rac3.	Western analysis, Rac1 and Rac3 activity assays, invasion, and migration assays completed.	Metastasis analyses being conducted.
3. Creation of a fluorescence illumination system for macro image analysis		Currently under analysis

Reportable Outcomes

The following manuscripts are in preparation:

1. Baugher, P., Krisnhamoorty, L., Price, J., and Dharmawardhane, S. Analysis of Rac1 and Rac3 Function in Breast Cancer Progression in metastatic variants of MB-435 Human Breast Cancer Cells. For submission to Cancer Research.
2. Hoffmeyer, M., Lacy, A.,Wall, K., Richards-Kortum, R., and Dharmawardhane, S. Adaptation of a fluorescence illumination system for analysis of the effects of breast cancer therapeutics by *in situ* image analysis of cancer progression in nude mice. For submission to Neoplasia.

Published Abstract:

Baugher, P., Krishnamoorty, L.. Dharmawardhane, S., The role of Rac1 and Rac3 in the metastatic progression of human breast cancer. Mol. Biol. Cell, 14: 49a. Annual meeting of the American Society of Cell Biology, San Francisco, CA, Dec 13-17, 2003.

Conclusions

The experiments proposed for the first two year in the statement of work has been accomplished.

As proposed in our task 1 for the first 18 months;,

A. Expression vectors containing the following mutations Rac3 and PAK have been created:

- I. Rac3(F28L) : dominant active fast cycler
- II. Rac3(G12V): dominant active
- III. Rac3(T17N): dominant negative
- IV. PAK1(83-149): autoinhibitory domain (AID)
- V. PAK1(83-149, L107F): ineffective AID

B. Stable human breast cancer cell lines containing the following mutations have been constructed:

- I. MDA-MB-435 highly metastatic breast cancer cell variant expressing dominant negative form of Rac3, Rac3(T17N).
- II. MDA-MB-435 highly metastatic breast cancer cell variant expressing dominant negative form of Rac1, Rac1(T17N).
- III. Hs578t non-metastatic breast cancer cell line expressing active fast cycling form of Rac3, Rac3(F28L).
- IV. A low metastatic variant of the MB-435 cell line (MB-435 Br) expressing Rac3(F28L), a highly active form of Rac3.
- V. MB-435 Br breast cancer cell line expressing dominant active Rac3, Rac3(G12V).
- VI. MB-435 Br breast cancer cell line expressing dominant active Rac1, Rac1(G12V).

C. We have completed the proposed *in vitro* characterization of the transfected cells expressing mutant Rac1 and Rac3.

- I. Western blotting of cell lysates with anti myc and anti Rac antibodies.
- II. Assays to determine activation status of mutant Rac3 proteins using the specific active Rho GTPase binding domain of PAK.
- III. Fluorescence microscopy to investigate changes in actin structures.
- IV. Invasion and migration assays in response to extracellular matrix components.

Task 2. Analyze the invasive behavior of the cell lines expressing Rac3 and PAK1 mutants in live mouse tumors (Months 18-30).

a. We are close to completion of the fluorescence confocal microscope to specifically image FP-tagged cells inside live mouse tumors.

b. We are analyzing the tumorigenic and metastatic efficiency of mutant cell lines in nude mice using an illumination system and have initiated implementation of optical and digital methods to image, analyze, and quantitate the invasive capabilities of the different cell lines *in vivo*.

Therefore, we have successfully completed a major proportion of the experiments proposed for the first 24 months of study. The data gathered from this initial phase agrees with our hypothesis that Rac3 is important for breast cancer metastasis.

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